PERPUSTAKAAN KAMPUS KESIHATAN
UNIVERSITI SAINS MALAYSIA

RUJUKAN

LAPORAN AKHIR PROJEK PENYELIDIKAN
R & D JANGKA PENDEK

UNIVERSITI SAINS MALAYSIA
DITERIMA
03 FEB 2005
Bahagian R & D
Pusat Pengajian Sains Perubatan

"*In vivo* toxicity study of a plant *Phyllanthus amarus*, in rat"

Investigators:

Dr. K.N.S.Sirajudeen
Prof. Madya Dr. Siti Amrah Sulaiman
Prof. Madya Dr. M.Madhavan
Prof. Madya Dr.Zabidah Ismail

Grant No: 304/PPSP/6131166

BAHAGIAN PENYELIDIKAN & PEMBANGUNAN
CANSELORI
UNIVERSITI SAINS MALAYSIA

Laporan Akhir Projek Penyelidikan Jangka Pendek

1) Nama Penyelidik: **Dr. K.N.S.Sirajudeen**

Nama Penyelidik-Penyelidik Lain (Jika berkaitan) : **PM. Dr. Siti Amrah Sulaiman**

PM. Dr. M. Madhavan

PM. Dr. Zabidah Ismail

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2) Pusat Pengajian/Pusat/Unit : Pusat Pengajian Sains Perubatan

3) Tajuk Projek: **"*In vivo* toxicity study of a plant *Phyllanthus amarus*, in rat"**

(Kajian tosiksiti in vivo *Phyllanthus amarus* terhadap tikus)

BAHAGIAN PENYELIDIKAN
PUSAT PENGAJIAN SAINS PERUBATAN

SALINAN :

- [ ] Bhg. Penyelidikan, PPSP
- [x] Perpustakaan Perubatan, USMKK
- [ ] RCMO

Tandatangan : ..... Tarikh : 2/5/06

4) (a) Penemuan Projek/Abstrak

(Perlu disediakan makluman di antara 100 – 200 perkataan di dalam Bahasa Malaysia dan Bahasa Inggeris. Ini kemudiannya akan dimuatkan ke dalam Laporan Tahunan Bahagian Penyelidikan & Pembangunan sebagai satu cara untuk menyampaikan dapatan projek tuan/puan kepada pihak Universiti).

Separate sheets attached

"Kajian tosiksiti in vivo *Phyllanthus amarus* terhadap tikus"

ABSTRAK

Pengenalan
Di Malaysia dan juga di negara lain, *Phyllanthus amarus* (*P. amarus*), sejenis spesis pokok dari keluarga Euphorbiaceae digunakan oleh pengamal perubatan tradisional bagi merawat jaundis dan penyakit lain. Adalah dilaporkan efikasi spesis ini berbeza dari segi kawasan geografi dan kepelbagaian dan perbezaan ini banyak dikaitkan dengan kandungan bahan di dalamnya. Bagaimanapun, buat masa ini tiada kajian toksiksiti yang lengkap dengan dokumentasi dijalankan di Malaysia.

Objektif
Tujuan kajian awal ini ialah untuk menentukan kesan toksik, jika ada, juga profil keselamatan ekstrak daun berakues *P. amarus* yang diberikan secara oral ke atas hepar tikus dengan menaksirkan perubahan morfologi, biokimia dan histologi.

Bahan dan metod
Tikus Sprague-Dawley (berat 180-230 g) digunakan sebagai model haiwan untuk kajian ini. Untuk kajian toksiksiti akut, tikus jantan diberikan ekstrak daun akues *P. amarus* (5g/kg) dan untuk kajian toksiksiti kronik, tikus jantan dan betina diberikan dos 100g, 400g dan 800g/kg selama 6 minggu. Haiwan kawalan juga disediakan tanpa diberikan sediaan ekstrak. Berat badan tikus diambil pada permulaan kajian dan berskala sehingga tamat kaiian. Selepas masa kajian (6 minggu), haiwan akan dikorbankan dan homogenat hepar tikus dianalisa untuk mendapatkan sebarang petanda biokimia kecederaan hepar, alanin transaminas (ALT), aspartat transaminas (AST), alkalin fosfatas (ALP), laktat dehidrogenas (LDH) dan jumlah protein serum. Bahagian hepar juga diambil untuk kajian histologi – mikroskopi ringan, kajian antigen nuklear sel proliferatif (PCNA) dan kajian apoptik dengan menggunakan Tag kit apop.

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Pemberian akut ekstrak *P. amarus* (5g/kg) tidak memberikan sebarang tanda toksiksiti atau kematian. Manakala kajian toksiksiti kronik tiada menunjukkan perbezaan signifikan ($P>0.05$) dari segi berat badan juga petanda biokimia (ALT, AST, ALP, LDH dan jumlah protein) di antara haiwan kawalan dengan haiwan yang diberikan ekstrak *P. amarus* sepanjang masa kajian. Ekstrak *P. amarus* yang tidak toksik dibuktikan oleh kajian histologi i.e. tiada kelihatan perbezaan di antara tikus kawalan dengan tikus yang memakan ekstrak *P. amarus*.

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Pemberian akut ekstrak *P. amarus* pada dos 5g/kg tidak memberikan kesan toksik kepada hepar tikus. Kajian toksiksiti kronik pula menunjukkan tiada toksiksiti kumulatif seperti yang ditunjukkan oleh tiada perubahan signifikan ke atas parameter yang dikaji, juga keputusan kajian histologi.

Kata kunci: *Phyllanthus amarus*, ekstrak akues, hepar, toksiksiti, enzim petanda, kajian histologi

ABSTRACT

Introduction: *Phyllanthus amarus (P. amarus)*, a plant species of *Euphorbiaceae* family, is used as a folk medicine for jaundice and other diseases in Malaysia and other countries. The variation in their efficacy with geographical location and varieties has been reported which has been attributed to their constituent composition. But, so far no toxicity studies have been carried out on this plant with clear documentation, especially with those plants growing in Malaysia.

Objective: The aim of this preliminary study was to determine the toxic side effects of aqueous extract of leaves of *P. amarus* (grown in Malaysia) following oral administration in rats by assessing the morphological, biochemical and histological changes.

Materials & Methods: Sprague-Dawley rats weighing (180-230 grams) were used as animal models in this study. Aqueous extract of leaves of *P. amarus* was administered orally to male rats for acute toxicity study (5 gram / Kg body weight) and to the male and female rats for chronic toxicity study (at the doses of 100, 400 and 800 mg/ Kg body weight/day for six weeks). Control of male and female rats was also maintained without plant extract administration. Body weight of the rats was taken initially and periodically once a week, till the end of experimental period. At the end of experimental period (6 weeks), the rats were sacrificed and analyzed for biochemical markers of liver injury - alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and total protein in the serum collected and the marker enzymes were assayed in the homogenates of the rat liver. Liver sections were taken for histological studies; Light microscopy, proliferative cell nuclear antigen (PCNA) study and apoptotic study by using ApopTag kit .

Results: Acute admininstration of *P. amarus* extract, orally, even at a dose of 5 gram/Kg body weight) did not produce any signs of toxicity or mortality. In the chronic study, no significant differences ($p>0.05$) were observed between the control and *P. amarus* extract administered rats in the body weight gain during the experimental period as well as in the biochemical markers analyzed (ALT, AST,ALP,LDH and total protein) in serum and liver homogenates. The non-toxic nature of *P. amarus* extract administration was confirmed by histological studies i.e., no observable changes were found between control and *P.amarus* extract administered rats.

Conclusion: Acute oral administration of *P.amarus* extract is non-toxic to the rat liver, even at a dose of 5 gram /kg body weight. The chronic toxicity study of *P. amarus* extracts administration showed the absence of cumulative toxicity as reflected by the non-significant change in the parameters studied as well as from the results of the histological studies.

Key words: ***Phyllanthus amarus*, Aqueous extract, Liver, Toxicity, Marker enzymes, Histological studies**

(b) Senaraikan Kata Kunci yang digunakan di dalam abstrak:

Bahasa Malaysia	Bahasa Inggeris
Phyllanthus amarus,	*Phyllanthus amarus*,
ekstrak akues	Aqueous extract
hepar	Liver
toksiksiti	Toxicity
enzim petanda	Marker enzymes
kajian histologi	Histological studies

5) Output Dan Faedah Projek

(a) Penerbitan (termasuk laporan/kertas seminar)
(Sila nyatakan jenis, tajuk, pengarang, tahun terbitan dan di mana telah diterbit/dibentangkan).

The manuscript entitled " Toxicological investiation of aqueous extract of leaves of *Phyllanthus amarus* in rat" (herewith attached) is going to be sent for publication in *Journal of Ethno Pharmacology*

An abstract from this work will be submitted for presentation in the coming 10th National Conference on Medical Sciences, PPSP, USM

(b) Faedah-Faedah Lain Seperti Perkembangan Produk, Prospek Komersialisasi Dan Pendaftaran Paten.
(Jika ada dan jika perlu, sila guna kertas berasingan)

- Tiada -

(c) Latihan Gunatenaga Manusia —

i) Pelajar Siswazah: .....

ii) Pelajar Prasiswazah: .....

iii) Lain-Lain : .....

6. Peralatan Yang Telah Dibeli:

Tiada

UNTUK KEGUNAAN JAWATANKUASA PENYELIDIKAN UNIVERSITI

T/TANGAN PENGERUSI
J/K PENYELIDIKAN
PUSAT PENGAJIAN

Professor Zabidi Azhar Mohd. Hussin
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KELANTAN, MALAYSIA.

UNIVERSITI SAINS MALAYSIA
JABATAN BENDAHARI
KUMPULAN WANG PENYELIDIKAN GERAN USM(304)
PENYATA PERBELANJAAN SEHINGGA 31 JANUARI 2005

Jumlah Geran:	RM	19,800.00	Ketua Projek:	DR. K.N.S. SIRAJUDEEN
Peruntukan 2002 (Tahun 1)	RM	0.00	Tajuk Projek:	In Vivo Toxicity Study of a Plant Phyllanthus amarus, in Rat
Peruntukan 2003 (Tahun 2)	RM	0.00		
Peruntukan 2004 (Tahun 3)	RM	0.00	Tempoh:	01 Jul 01 - 14Apr 04
			No.Akaun:	304/PPSP/6131166

Kwg	Akaun	PTJ	Projek	Donor	Peruntukan Projek	Perbelanjaan Tkumpul Hingga Tahun Lalu	Peruntukan Semasa	Tanggungan Semasa	Bayaran Tahun Semasa	Belanja Tahun Semasa	Baki Projek
304	11000	PPSP	6131166			-	-	-	-	-	-
304	13000	PPSP	6131166			-	-	-	-	-	-
304	15000	PPSP	6131166			-	-	-	-	-	-
304	21000	PPSP	6131166		910.00	395.00	515.00	-			515.00
304	22000	PPSP	6131166			-	-	-		-	
304	23000	PPSP	6131166		300.00	4.73	295.27	-			295.27
304	26000	PPSP	6131166		500.00		500.00	-	-	-	500.00
304	27000	PPSP	6131166		12,370.00	14,933.25	(2,563.25)	3,757.00		3,757.00	(6,320.25)
304	29000	PPSP	6131166		2,220.00	702.00	1,518.00	-		-	1,518.00
304	35000	PPSP	6131166		3,500.00	-	3,500.00	-		-	3,500.00
					19,800.00	16,034.98	3,765.02	3,757.00	-	3,757.00	8.02

TABLE OF CONTENTS

"Kajian tosiksiti in vivo *Phyllanthus amarus* terhadap tikus"

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Key words: *Phyllanthus amarus*, Aqueous extract, Liver, Toxicity, Marker enzymes, Histological studies

1. INTRODUCTION

Herbal medicines has been used since ancient times. Most of the population of the underdeveloped and developing countries depend on some form of traditional medicine. Malaysia is one of the important nations of biodiversity in the world and enriched with natural plant resources(Muhamad bin Zakaria and Mustafa Ali Mohd, 1994).

One of the plant genuses widely used traditionally for the treatment of different diseases is *Phyllanthus* (Family: *Euphoribaceae*) and is distributed in most tropical and subtropical countries and comprise approximately of 550-750 species throughout the world. Among them, one of the most studied species is *P.amarus* and is widely used in Malaysia and other countries (Calixto *et al.*, 1998; Muhamad bin Zakaria and Mustafa Ali Mohd, 1994).

Some of the vernacular names of the plant include: Dukong anak (Malay), Kilanelli (Tamil), Bhumyamalakai (Sanskrit), Chanca piedra, Quebra pedra (Brazil).

P.amarus has bitter, astringent, cooling, diuretic, stomachic, antiseptic, antiviral, antidiabetic, hypotensive, antinociceptive, febrifuge properties and is traditionally used in the treatment of jaundice, diarrhea, dysentery, diabetes, fevers, uro-genital diseases, ulcers and wounds (Santos *et al,* 1995; Calixto *et al*, 1998). Recent years, a growing interest shown towards the *Phyllanthus* with respect to their potential on management of several diseases (Odetola and

Akojenu, 2000; Rajeshkumar *et al.*, 2002; Srividya and Periwal, 1995) More research was done on its antiviral effect. Some reports have shown the antiviral effect of this plant by reducing the dectectable hepatitis B surface antigen (HbsAg) of HBV positive patients (Thyagarajan *et al.*,1988; Ott et al., 1997). But other studies like from China, Thailand , India showed the failure of P.amarus in eradicating the HbsAg in patients with chronic hepatitis B virus (Leelarasamee *et al*, 1990; Wang *et al*, 1991, Doshi *et al*, 1997). The variation in the clinical effect of these studies has been attributed to many factors such as different species, differences in growing condition and different processing methods (Thyagarajan *et al.*, 2002; Wang, 2000).

Usually herbal medicines are widely perceived by the public as being natural, healthful and free from side effects, but that is not 100 % true. Plants contain hundreds of constituents and some of them may elicit a toxic side effects. A number of studies exist reporting the toxic effect of herbal medicines (Shaw *et al.,* 1997; Kaplowitz, 1997; Calixto, 2000). Therefore efficacy and safety study should be performed in these herbs.

Eventhough a large number of clinical trials has been done on P.amarus and reporting their benefits, so far no systematic toxicological investigation has been reported on this plant, especially the P. amarus growing in Malaysia.

Since the efficacy of Phyllanthus species varies with geographical location and varieties and because of their constitutent compostion variation, the

P.amarus grown in Malaysia (widely used here as the folk medicine for jaundice) has to be ascertained for their biosafety by conducting acute and chronic toxicity study .

So the aim of this preliminary study was an attempt to determine the toxic effects of aqueous extract of leaves of *P. amarus* (grown in Malaysia) following oral administration in rats by assessing the morphological, biochemical and histological changes.

2 MATERIALS AND METHODS

2.1 Plant material

P.amarus was collected from MARDI (Malaysian Agricultural Research and Development Institute, Telung, Bachok, Kelantan) during the months of July-October and identified by FRIM (Forest Research Institute of Malaysia, Kepong, Selangor). The aqueous extraction of P.amarus leaves was carried out at the Department of Pharmacology, School of Medical Sciences, Universiti Sains Malaysia.

2.2 Preparation of plant extract

The plant material was cleaned and the leaves were separated, oven dried at 50°C. Then macerated into dry powder. Approximately 4.5 grams of fine leaves powder of *P.amarus* were extracted with distilled water (2:25) using soxhlet apparatus. After extraction, concentrated by rotary evaporator at 65°C. Then transferred into a suitable container and freeze dried by freeze dryer. The yield of the final crude aqueous *P.amarus* extract was 6-9% (~380 grams). The dried extract was stored in a desicator until its use. The extract was dissolved in distilled water to the desired concentration just before the study.

2.3 Animal and experimental design

Male and female Sprague-Dawley rats weighing (180-220 grams) were used as the experimental animal in this study and were obtained from Animal House Facility Unit, Health Campus, Universiti Sains Malaysia and acclimatized for one week prior to start the experiment. Animals were

housed in a standard cages at a temperature of 23±2°C, 45-55 % relative humidity with a 12h light/12h dark cycle. The animals were fed with commercial pellet diet and water *ad libitum*.

Our study protocol was approved by the animal ethical committee, Health Campus, Universiti Sains Malaysia.

Two types of studies were carried out (WHO, 1993; Arnold, 1990) Acute and chronic study and in both studies the rats were administered with P.amarus extract by gavage (orally).

2.4 Acute study

Aqueous leaves extract of P.amarus at a single dose of 5g/Kg body weight was administered orally to two male rats. Another two male rats served as control (without *P.amarus* extract administration). The animals were observed carefully for any visible signs of toxicity and mortality immediately after dosing, at 4h, 24h intervals, during the recovery period of 48h and twice daily upto 14 days. After 14 days, the rats were sacrificed under ether anesthesia. A thorough autopsy was carried out and all organs were observed for any macroscopic changes.

2.5 Chronic study

2.5.1 Animal groups

For the chronic study, the animals were grouped (ten rats in each group) as follows

Male rats	Female rats
Group I – Control Group II, III & IV – Rats administered with *P.amarus* extract at the doses of 100,400 and 800 mg/Kg body wt/day for 6 weeks by gavage, respectively	Group A – Control Group B, C & D – Rats administered with *P.amarus* extract at the doses of 100,400 and 800 mg/Kg body wt/day for 6 weeks by gavage, respectively

The animals were observed daily for any signs of morbidity and mortality and their body weights were measured periodically in the experimental period.

2.5.2 Collection of serum and liver samples for anlaysis

At the end of the experimental period (6 weeks), after an overnight fasting, both the male and female rat controls and P.amarus extract administered groups were sacrificed by decapitation. Blood was collected, allowed to clot and then centrifuged at 3,000 rpm for 15 minutes. Serum samples were separated and used for biochemical analysis. The samples were stored at -80°C, if not used immediately.

Liver tissues were excised and washed in ice-cold saline and an weighed amount was homogenized with 0.1M Tris buffer at pH 7.4 under cold

condition. The supernatant of the liver homogenates was used for the biochemical estimations.

A portion of the liver tissue of all the (control and *P.amarus* extract administered) groups were fixed in 10% formal saline for the histological studies.

2.5.3 Biochemical analysis

In the collected serum, the total protein and the activities of the liver marker enzymes such as ALT,AST,ALP and LDH were assayed by using standard kit (ALT Randox kit , Randox Total protein biuret reagent- Randox Laboratories Ltd, UK; AST,ALP and LDH Roche Kits - Roche diagnostics, GmbH, Germany) in a Hitachi-912 autoanalyser available in our department of chemical Pathology, School of Medical Sciences, Universiti Sains Malaysia.

Similarly the activities of the marker enzymes – ALT,AST, ALP and LDH were assayed in the liver homogenates prepared.

2.5.4 Histological studies

This study was carried out in the department of Pathology, School of Medical Sciences, Universiti Sains Malaysia.

A portion of all the liver specimens fixed in 10% formal saline were processed routinely overnight using histokinette. Then they were embedded in paraffin was. Three sections, each four micron in thickness were cut from each paraffin block.

Light Microscopic study

One section from each sample was stained with Haematoxylin & Eosin (H&E) stain by the standard method for light microscopic histological examination

Proliferative Cell Nuclear Antigen (PCNA) study

Another section from each sample was stained with anti PCNA (DAKO, Denmark) using standard immunohistochemistry procedure and the positivity was visualized with the chromogen diaminobenzidine (DAB) (DAKO, Denmark) and expressed in percentage.

Apoptosis study

The third section from each sample was assessed for apoptosis. They were stained with ApopTag –Apoptosis dectection kit (Chemicon, USA) whereby the reagents are designed to label the free 3'OH DNA termini in situ with chemically labelled and unlabelled nucleotides. The nucleotides contained in the Reaction Buffer are enzymatically added to the DNA by terminal deoxynucleotidyl transferase (TdT). TdT catalyzes a template-independent addition of nucleotide triphosphates to the 3'OH ends of

double stranded or single stranded DNA. The incorporated nucleotides form an oligomer composed of digoxigenin-conjugated nucleotide and unlabelled nucleotide in a random sequence. The ratio of labelled to unlabelled nucleotide in ApopTag Peroxidase Kits is optimized to promote anti digoxigenin antibody binding. DNA fragments which have been labelled with digoxigenin-nucleotide are then allowed to bind an anti-digoxigenin antibody that is conjugated with to a peroxidase reporter molecule. The bound peroxidase antibody conjugate enzymatically generates a permanent, intense, localized stain from chromogenic substance, providing sensitive detection in immunohistochemistry. This mixed molecular biological-histochemical system allows fro sensitive and specific staining of very high concentrations of 3'OH ends that are localised in apoptotic bodies.

2.6 Statistical Analysis

The results of the biochemical analysis are expressed as mean ± S.D. for ten animals in each group. The difference between the control and P.amarus extract administered groups (for each sex of animals) have been analysed by student's t-test. p value <0.05 , considered as significant.

3. RESULTS

3.1 ACUTE STUDY

During the course of the acute study, no treatment related effect was observed on the general condition or behaviour of the experimental animals. All the rats appeared to be normal, survived during the experimental period and none of them showed any visible signs of toxicity. A thorough autopsy of the *P.amarus* administered rats revealed no treatment related macroscopic changes.

3.2 CHRONIC STUDY

3.2.1 Body weight

Mean body weight gain of the P.amarus extract administered groups (at the doses of 100,400 and 800 mg/Kg body weight/day) have shown no appreciable difference when compared to their control (both male and female rats) after 6 weeks duration of the study (Figure 1).

The macroscopic appearance of all the organs observed was found to be normal and there were no appreciable changes in the liver weight of *P.amarus* extract administered groups and control group (data not shown).

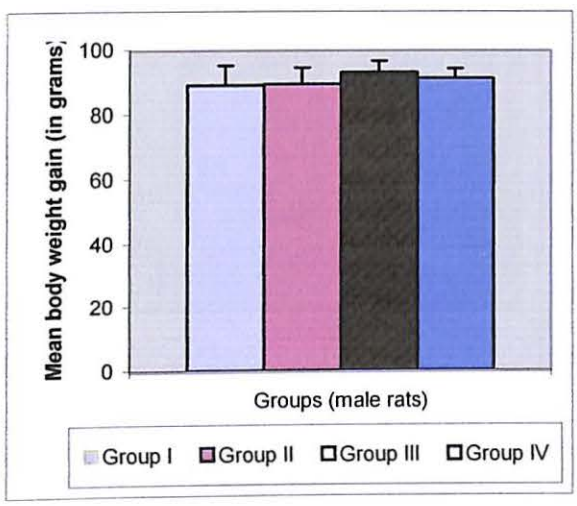
3.2.2 Biochemical study

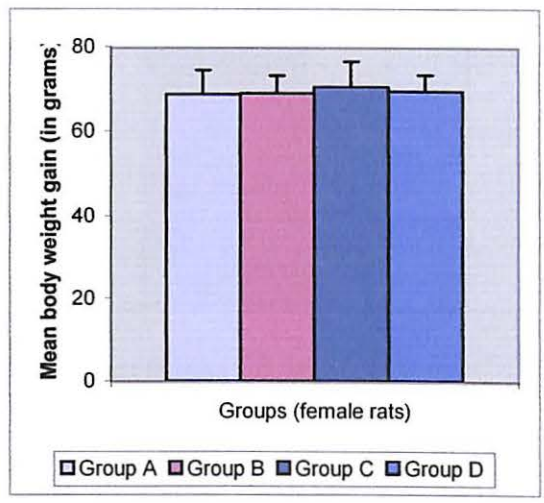
There were no significant differences ($p>0.05$) observed in the biochemical parameters studied (ALT,AST,ALP,LDH and total protein) in

the serum between controls and *P.amarus* extract administered groups (of both male and female) (Figure 2 & 3).

Similarly, the parameters assayed (ALT,AST,ALP and LDH) in the liver homogenates of controls and P.amarus extract administered groups also showed no significant difference ($p>0.05$) (Figure 4 & 5).

Fig. 1 : Mean body weight gain of control and *P. amarus* administered groups, Male and Female rats at the end of experimental period

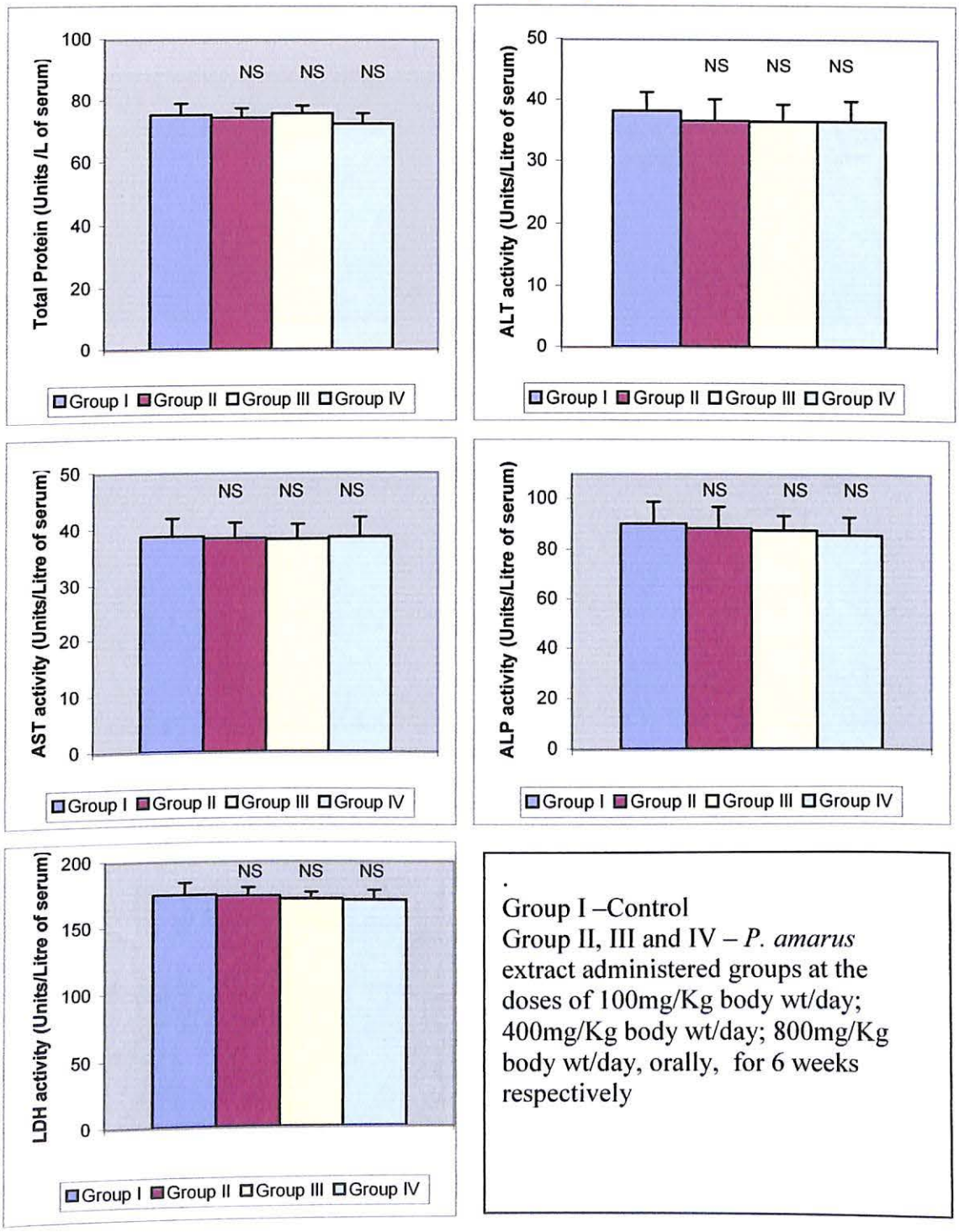




Groups (Male rats)	Groups (Female rats)
Group I –Control Group II, III and IV – *Phyllanthus amarus* extract administered groups at the doses of 100mg/Kg body wt/day; 400mg/Kg body wt/day; 800mg/Kg body wt/day, orally, for 6 weeks respectively	Group A –Control Group B,C and D – *P. amarus* extract administered groups at the doses of 100mg/Kg body wt/day; 400mg/Kg body wt/day; 800mg/Kg body wt/day , orally, for 6 weeks respectively

Values are mean ± S.D. for ten animals in each group & analyzed by Student's t-test. No significant changes in the mean body weight gain observed when *P.amarus* extract administered groups compared with control group (of both male and female rats).

Figure 2: Level of total protein and activities of serum marker enzymes of control and *P. amarus* extract administered groups (male rats)



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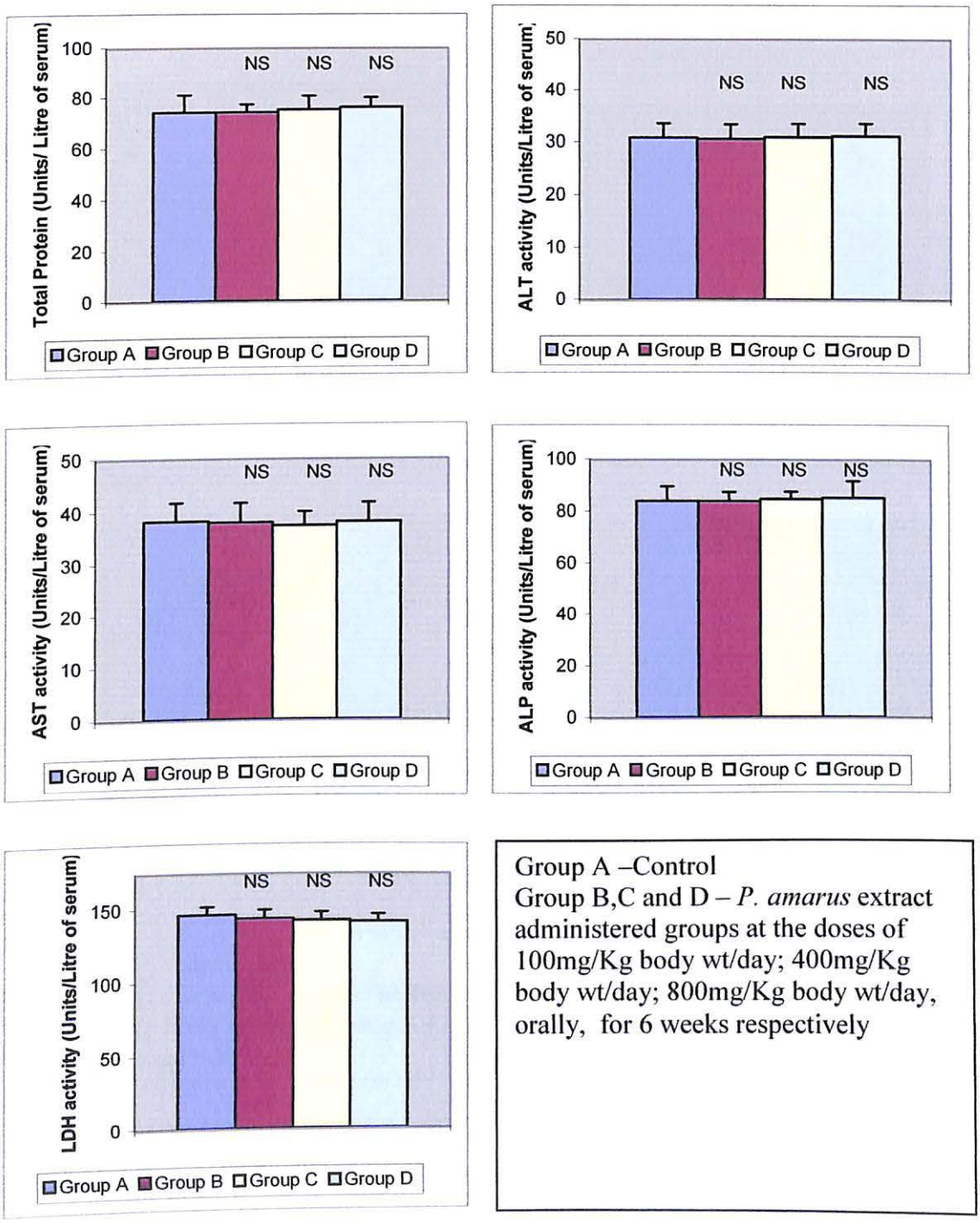
Group I –Control
Group II, III and IV – *P. amarus* extract administered groups at the doses of 100mg/Kg body wt/day; 400mg/Kg body wt/day; 800mg/Kg body wt/day, orally, for 6 weeks respectively

Values are mean ± S.D. for ten animals in each group and analyzed by Student's t-test. Group II,III and IV compared with Group I (control) and p value <0.05 considered significant.
^{NS} $p>0.05$- not significant

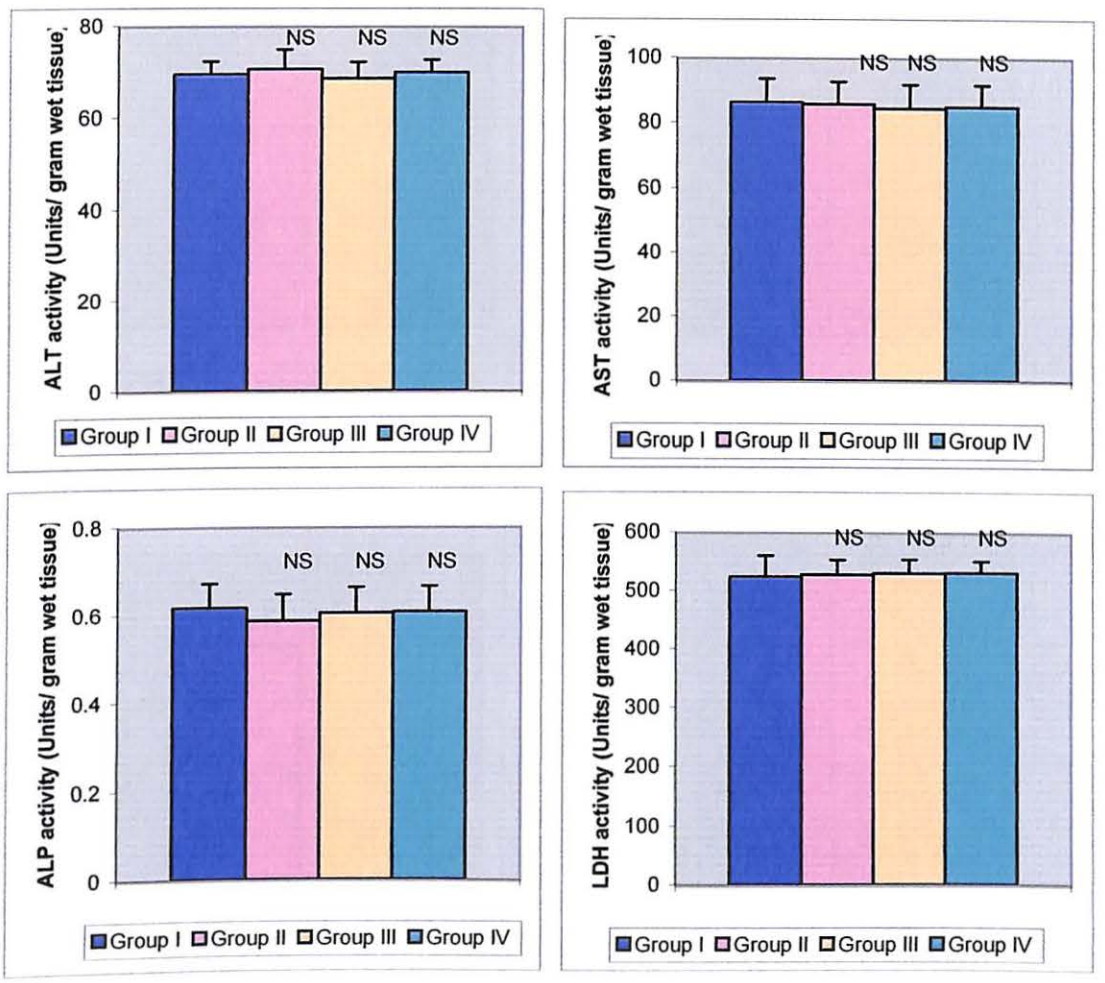
Figure 3: Level of total protein and activities of serum marker enzymes of control and *P. amarus* extract administered groups (female rats)



Group A –Control
Group B,C and D – *P. amarus* extract administered groups at the doses of 100mg/Kg body wt/day; 400mg/Kg body wt/day; 800mg/Kg body wt/day, orally, for 6 weeks respectively

Values are mean ± S.D. for ten animals in each group and analyzed by Student's t-test. Group B,C and D compared with Group A (control) and p value <0.05 considered significant.
NS $p>0.05$- not significant

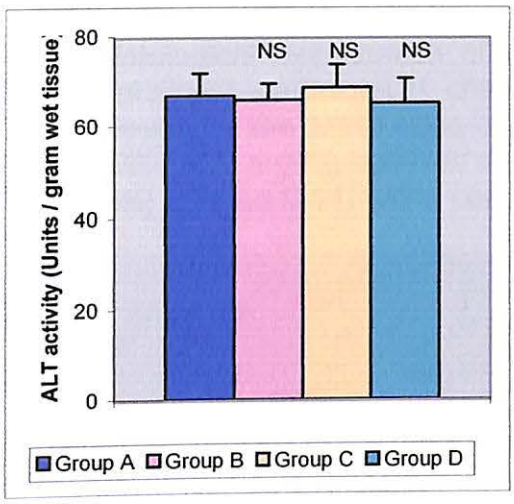
Figure 4: Activities of ALT, AST, ALP and LDH in the liver homogenates of control and *P.amarus* extract administered groups (male rats)

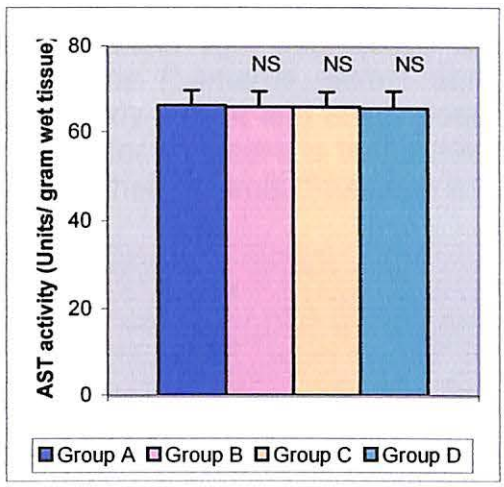


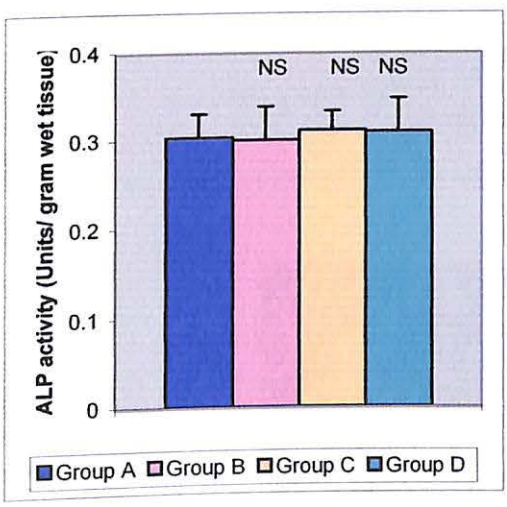
Group I –Control;
Group II, III and IV – *P. amarus* extract administered groups at the doses of 100mg/Kg body wt/day; 400mg/Kg body wt/day; 800mg/Kg body wt/day, orally, for 6 weeks respectively.

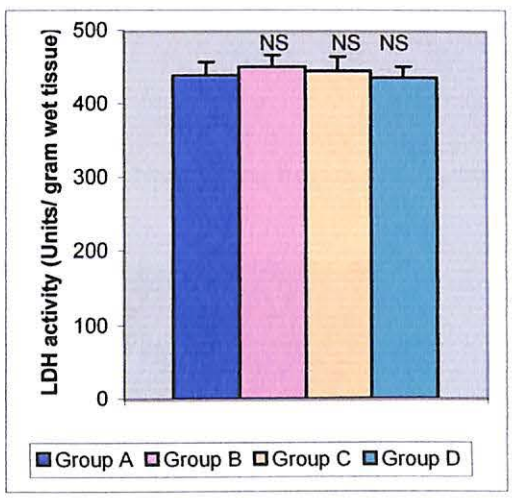
Values are mean ± S.D. for ten animals in each group and analyzed by Student's t-test.
Group II,III and IV compared with Group I (control) and p value <0.05 considered significant.
^{NS} p>0.05- not significant

Figure 5: Activities of ALT, AST, ALP and LDH in the liver homogenate of control and *P. amarus* extract administered groups (female rats)









Group A –Control;
Group B,C and D – *P. amarus* extract administered groups at the doses of 100mg/Kg body wt/day; 400mg/Kg body wt/day; 800mg/Kg body wt/day, orally, for 6 weeks respectively.

Values are mean ± S.D. for ten animals in each group & analyzed by Student's t-test.
Group B,C and D compared with Group A (control) and p value <0.05 considered significant.
^{NS} p>0.05- not significant

3.3 Histological study

3.3.3. Light microscopic study

Histological examination of the liver under light microscope showed no significant pathological changes in the *P.amarus* extract administered groups (at the acute dose of 5g/Kg body weight and at the doses of 100, 400 & 800 mg/Kg body wt/ day, orally, for six weeks to both male & female rats) (figures 8-14) when compared to their controls (Figures 6 & 7).

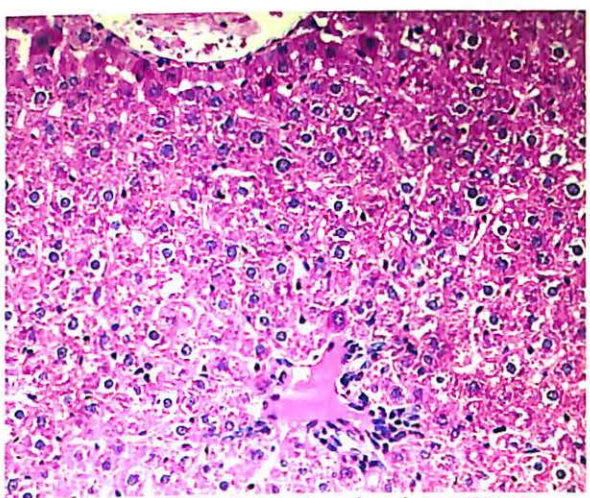


Fig. 6: Normal liver from control male rat (H&E)

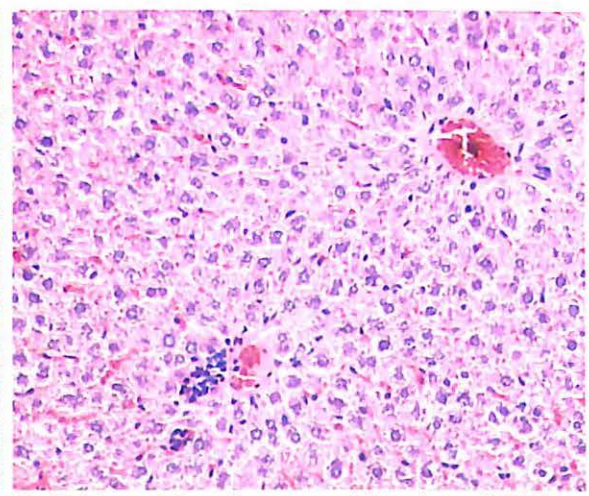


Fig.7: Normal liver from control female rat (H&E)

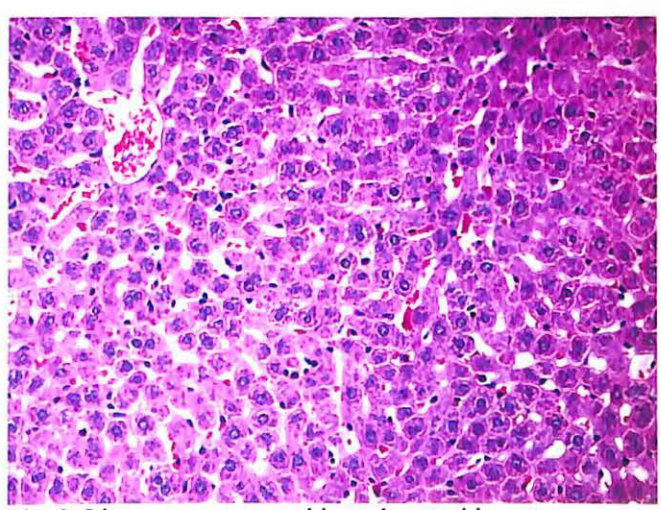


Fig. 8: Liver appears normal in male rat with acute administration of *P.amarus* extract 5g/Kg body wt. (H&E)

3.3.2 PCNA Study

Anti PCNA antibody was used to measure the proliferation of hepatocytes by immunohistochemistry. The cells that are in the proliferative pool shows brown colour staining (Figure 15). PCNA of liver from control rat was 1.9% (Figures 16 &17) and for the acute dose (5g/Kg body weight) *P.amarus* extract administered rat was 1.8% (Figure18). For the *P.amarus* extract administered groups at the doses of 100, 400 & 800 mg/Kg body wt/ day , orally, for 6 weeks (both male & female rat livers) showed PCNA of 0.2% -2.1% (Figures 19-24) which are similar to that of their controls.

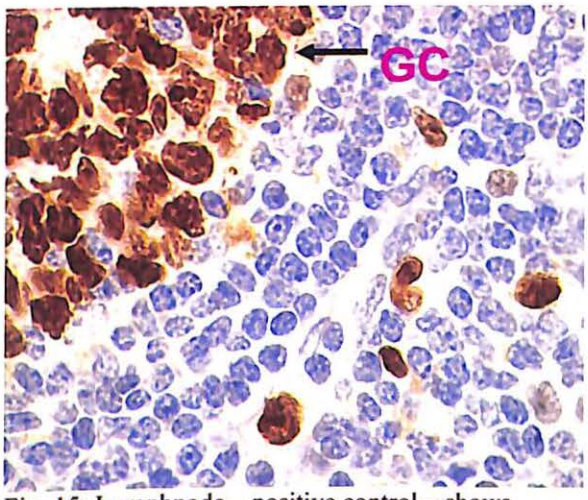


Fig. 15: Lymphnode – positive control – shows increased proliferation in the germinal centre (GC) (anti PCNA)

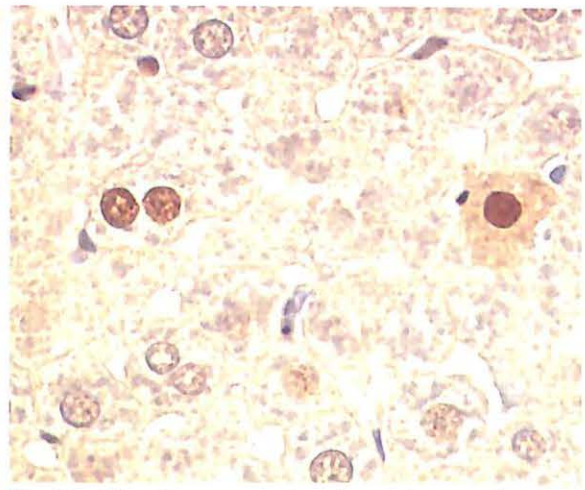


Fig.16: Liver from control male rat shows only occasional cells in proliferative cycle (anti PCNA)

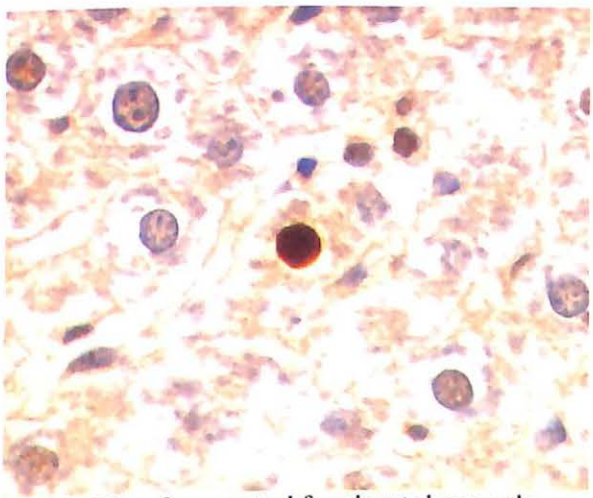


Fig.17: Liver from control female rat shows only occasional cells in proliferative cycle (anti PCNA)

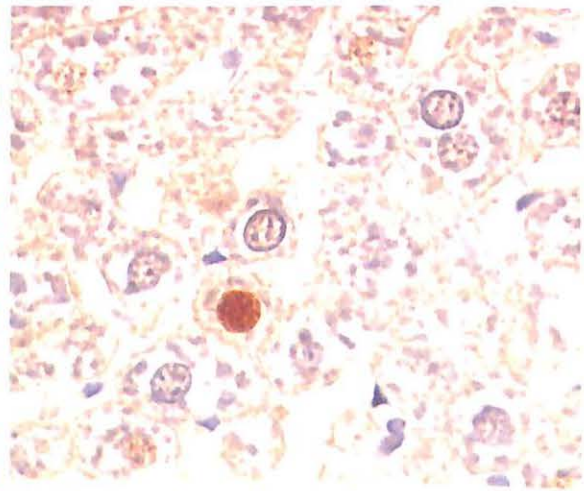


Fig.18: Liver from male rat with acute administration of *P.amarus* extract 5g/Kg body wt. shows only occasional cells in proliferative cycle (anti PCNA)

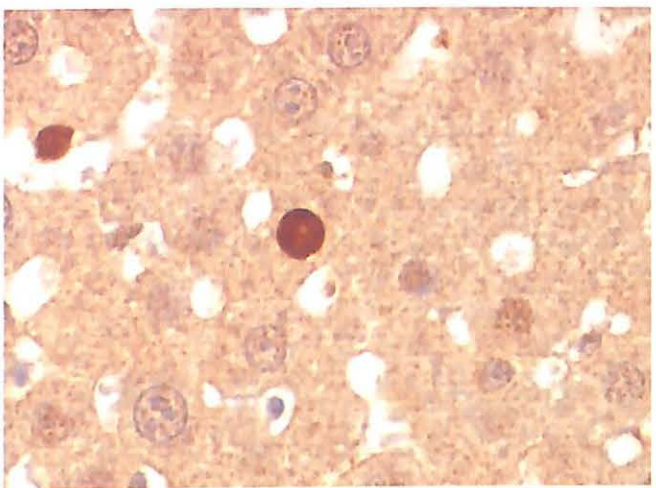


Fig.19: Liver from male rat administered with *P.amarus* extract 100 mg/Kg body wt/ day for 6 weeks shows only occasional cells in proliferative cycle (anti PCNA)

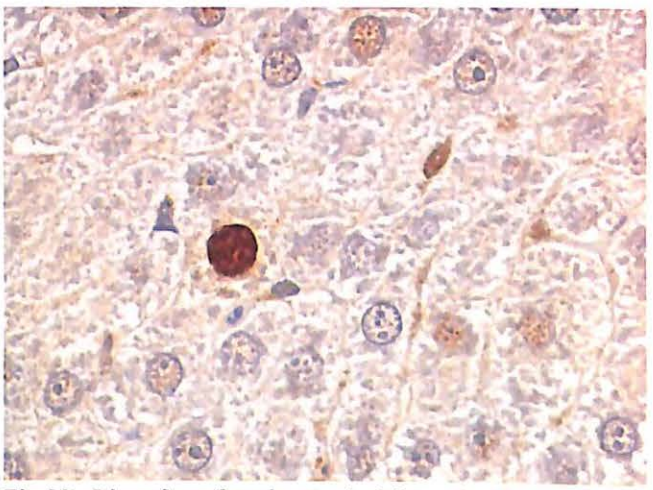


Fig.20: Liver from female rat administered with *P.amarus* extract 100 mg/Kg body wt/ day for 6 weeks shows only occasional cells in proliferative cycle (anti PCNA)

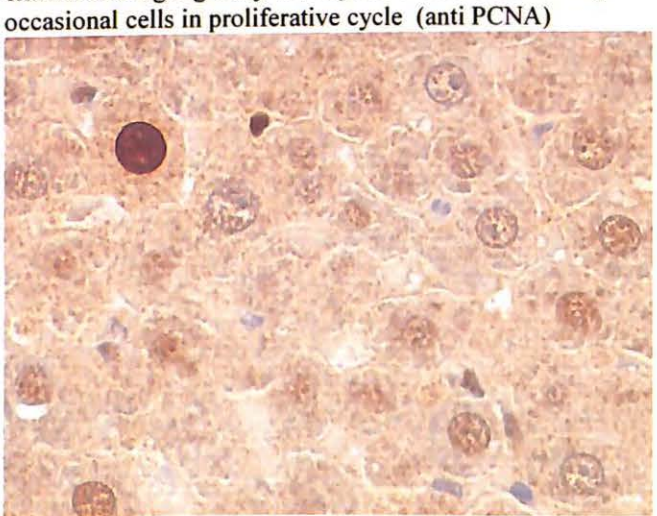


Fig.21: Liver from male rat administered with *P.amarus* extract 400 mg/Kg body wt/ day for 6 weeks shows only occasional cells in proliferative cycle (anti PCNA)

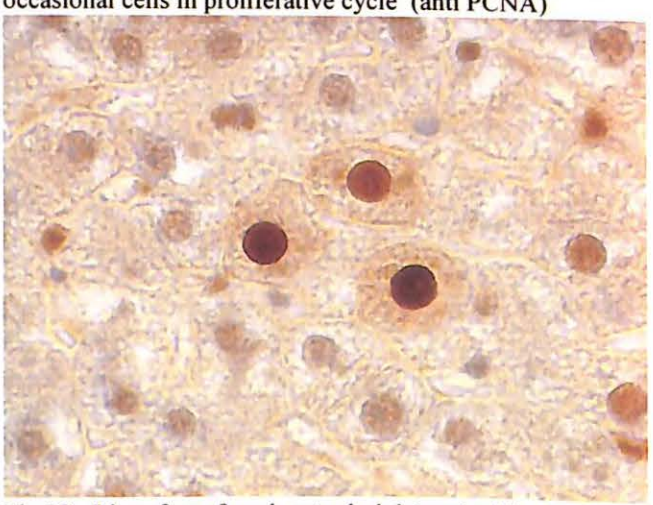


Fig.22: Liver from female rat administered with *P.amarus* extract 400 mg/Kg body wt/ day for 6 weeks shows only occasional cells in proliferative cycle (anti PCNA)

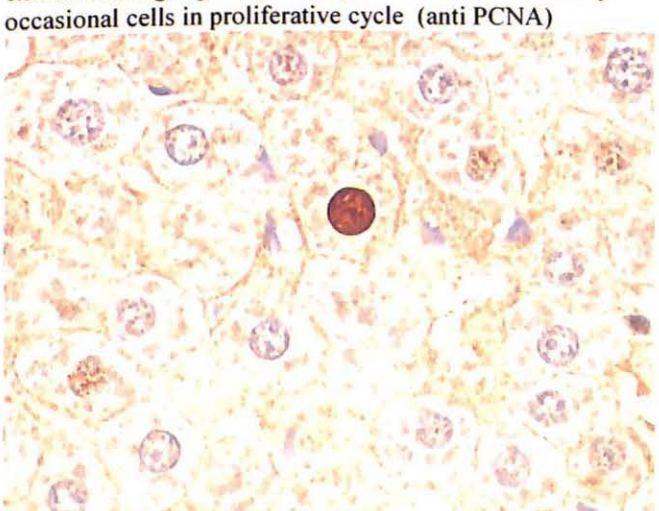


Fig.23: Liver from male rat administered with *P.amarus* extract 800 mg/Kg body wt/ day for 6 weeks shows only occasional cells in proliferative cycle (anti PCNA)

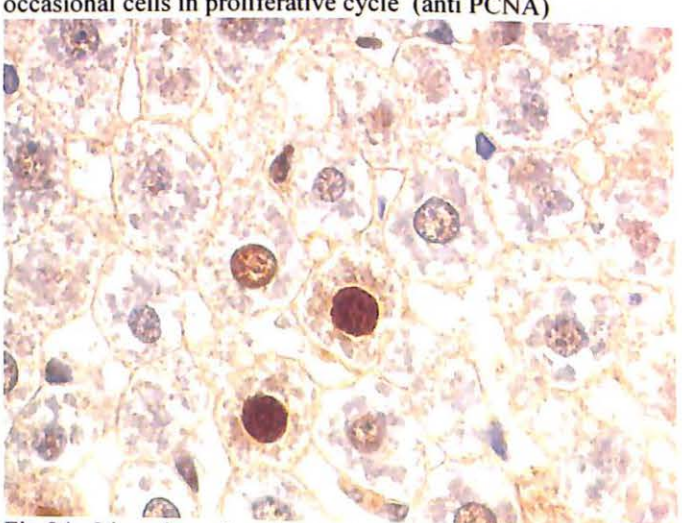


Fig.24: Liver from female rat administered with *P.amarus* extract 800 mg/Kg body wt/ day for 6 weeks shows only occasional cells in proliferative cycle (anti PCNA)

3.3.3 Apoptosis study

Apoptosis in the liver was measured in the hepatocytes using ApopTag Kit. The positivity was indicated by the brown colour in nucleus (Figure 25). Apoptosis in the livers from control (both male and female rats) were 0.2% (Figure 26 &27). Apoptosis in the livers from *P.amarus* extract administered groups were ranged from 0.2% to 1.5% (Figures 28-34) which are similar to that of their controls.

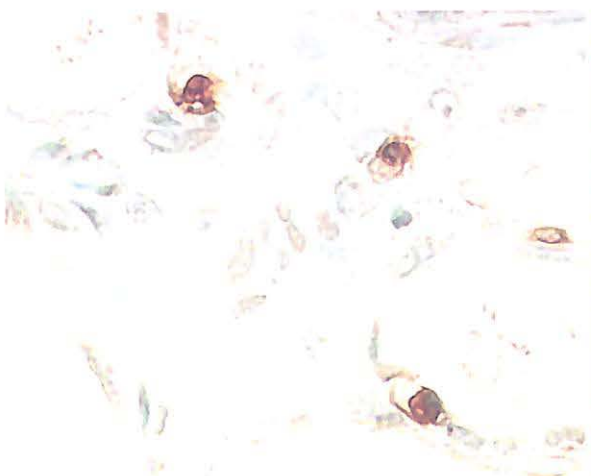


Fig. 25: Apoptotic bodies are stained brown in a positive control slide (ApopTag kit)

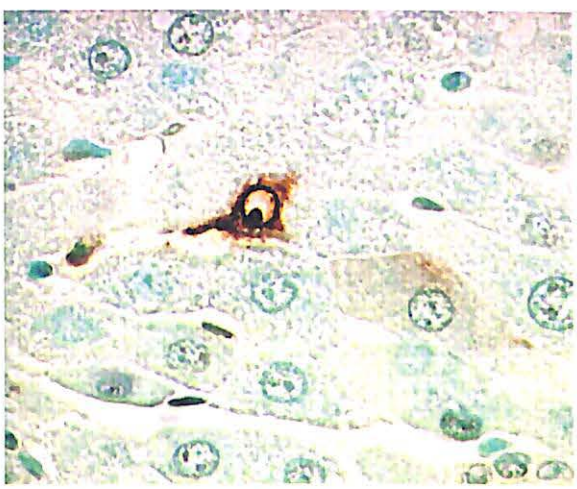


Fig. 26: Normal control male liver shows occasional apoptotic bodies (ApopTag kit)

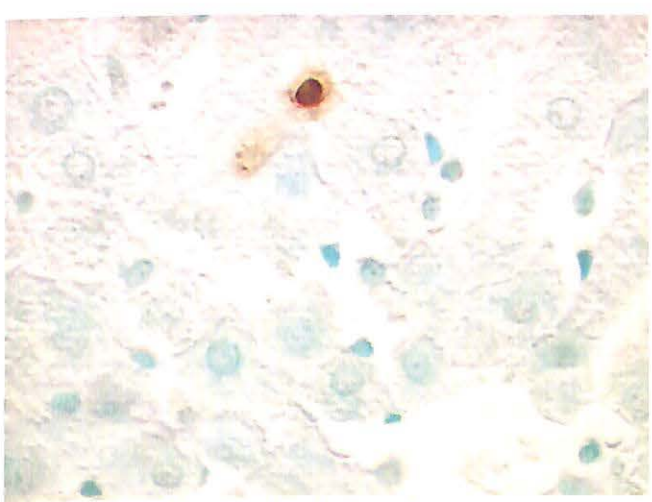


Fig. 27: Normal control female liver shows occasional apoptotic bodies (ApopTag kit)

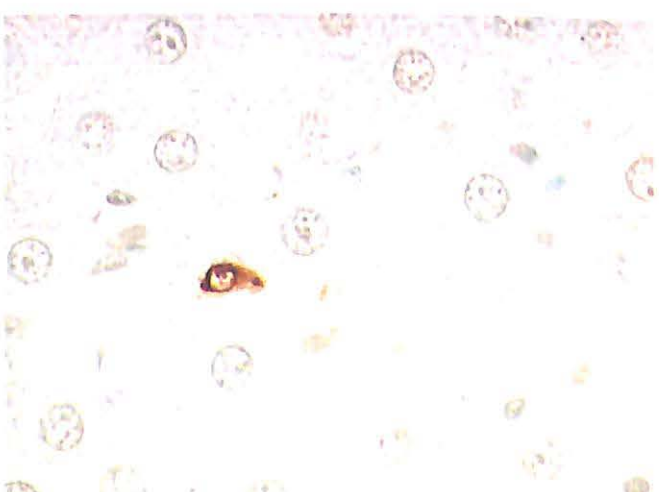


Fig. 28: Male rat liver administered with acute *P.amarus* extract at a dose of 5g/Kg body wt, shows occasional apoptotic bodies (ApopTag kit)

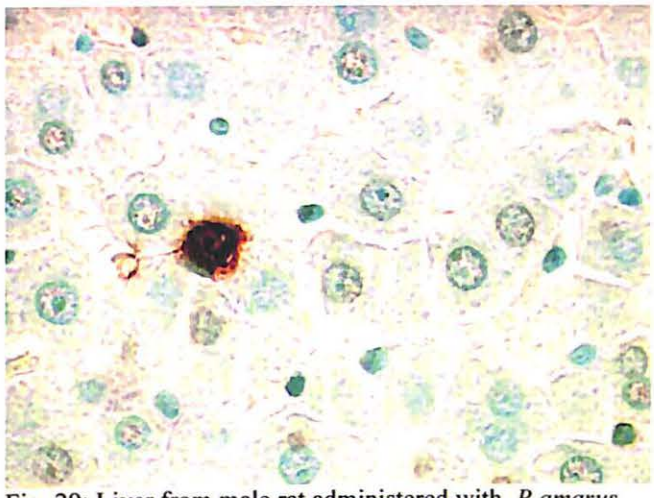


Fig. 29: Liver from male rat administered with *P.amarus* extract 100 mg/Kg body wt/ day for 6 weeks shows occasional apoptotic bodies (ApopTag kit)

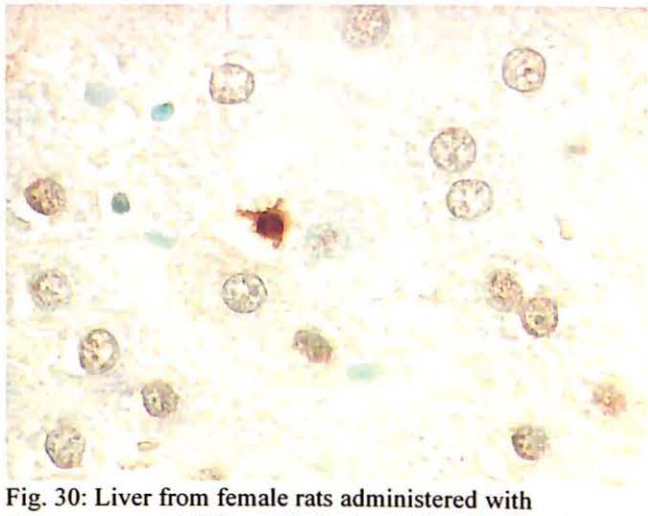


Fig. 30: Liver from female rats administered with *P.amarus* extract 100 mg/Kg body wt/ day for 6 weeks hows occasional apoptotic bodies (ApopTag kit)

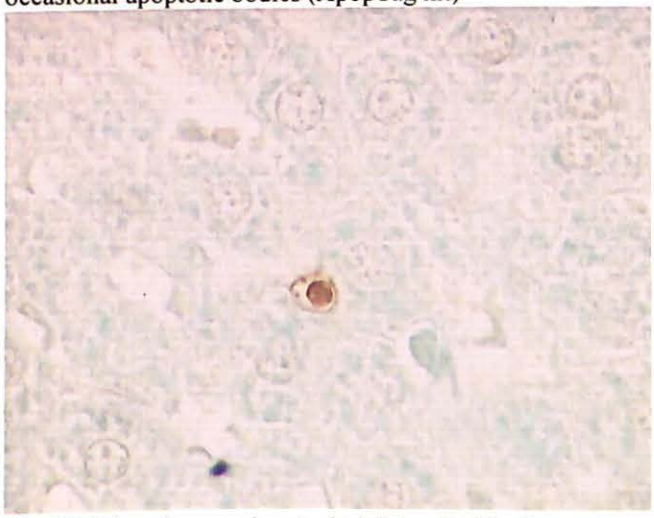


Fig. 31: Liver from male rat administered with *P.amarus* extract 400 mg/Kg body wt/ day for 6 weeks shows occasional apoptotic bodies (ApopTag kit)

Fig.32: Liver from female rat administered with *P.amarus* extract 400 mg/Kg body wt/ day for 6 weeks shows occasional apoptotic bodies (ApopTag kit)

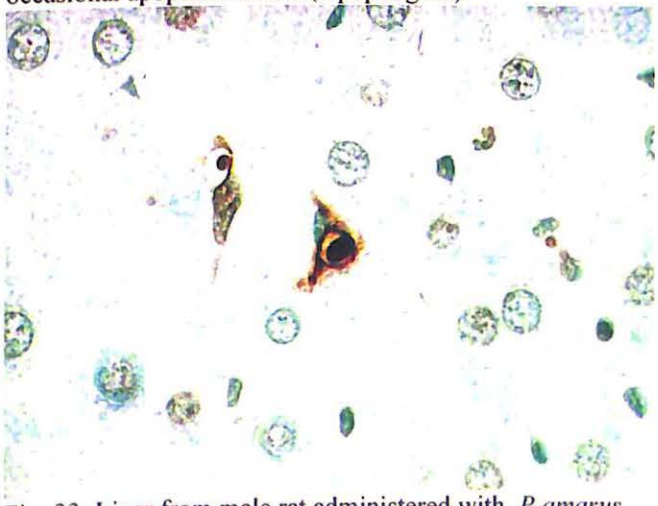


Fig. 33: Liver from male rat administered with *P.amarus* extract 800 mg/Kg body wt/ day for 6 weeks shows occasional apoptotic bodies (ApopTag kit)

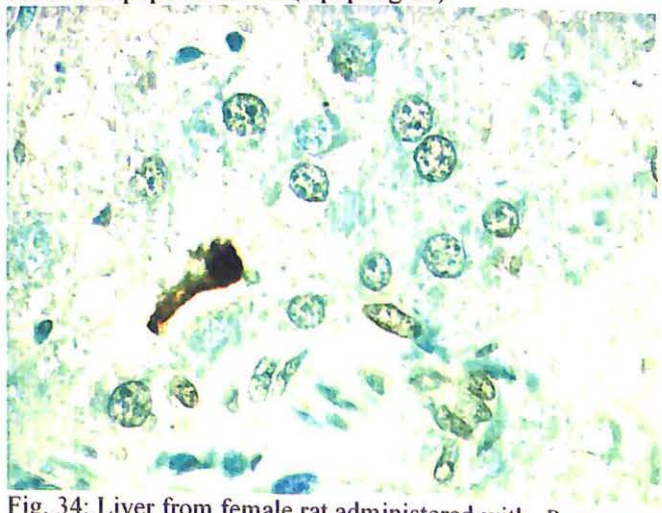


Fig. 34: Liver from female rat administered with *P.amarus* extract 800 mg/Kg body wt/ day for 6 weeks shows occasional apoptotic bodies (ApopTag kit)